Larval Behavioral, Morphological Changes, and Nematocyte Dynamics During Settlement of Actinulae of *Tubularia mesembryanthemum*, Allman 1871 (Hydrozoa: Tubulariidae)

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Abstract. The marine colonial hydroid *Tubularia mesembryanthemum* produces a morphologically unique dispersive stage, the actinula larva. Detailed observations were made on the behaviors and nematocyte dynamics of actinula larvae during attachment and morphogenesis by employing microscopic and time lapse video techniques. These observations produced four primary results. (1) Actinula larvae demonstrated two forms of attachment: temporary attachment by *atrichous isorhiza* (AI)—nematocysts discharged from the aboral tentacle (AT) tips—and permanent settlement by cement secretion from the columnar gland cells of the basal protrusion. (2) During larval settlement, numerous AIs were discharged from the AT tips with sinuous movement and rubbing of the tentacles onto the substrata, leading to "nematocyte-printing" around the settlement site. (3) Simultaneous with the discharge of the AIs, migration of *stenoteles, desmonemes,* and *microbasic mastigophores* occurred, resulting in a dramatic change of nematocyte composition in the ATs after larval settlement. This was in parallel with changes in larval behavior and the tentacle function. (4) Nematocyte-printing behavior during settle-
ment could be recognized as metamorphic behavior responsible for irreversible changes in AT function, from attachment to feeding and defense.

Introduction

Many marine sessile invertebrates produce planktonic or benthic larvae as a dispersive phase. These larvae develop to competent stages, attach to suitable substrata, and metamorphose into juveniles in response to certain environmental (physical, biological, and chemical) cues (Crisp, 1974, 1984; Chia and Bickell, 1978; Pawlik, 1992). Many marine colonial hydroids (Cnidaria) produce free-swimming planula larvae for dispersal. Attachment and metamorphosis of some hydroid planulae are induced by certain bacteria, various pharmacologically active compounds, or neurotransmitter peptides (Müller, 1985; Leitz and Müller, 1987; Berking, 1988; Leitz and Klingman, 1990; Leitz et al., 1994). The biochemical and physiological mechanisms involved in attachment and metamorphosis of *Hydractinia* have been described (Chia and Bickell, 1978; Berking, 1991; Leitz, 1993, 1997).

Marine hydrozoans in the genus *Tubularia* are widely distributed in shallow waters throughout the world (Petersen, 1990). Their relatively large polyps have been excellent subjects for biological studies in areas such as regeneration (Barth, 1940; Tardent and Eymann, 1958), early development of the gonophores (Brauer, 1891; Berrill, 1952; Nagao, 1965), growth in culture (Mackie, 1966), field ecology (Hughes, 1983; Östman et al., 1995), physiology (Josephson and Mackie, 1965; Neufeld et al., 1978; Michel and Case, 1986), and taxonomy (Tardent, 1980; Petersen, 1990).

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Abbreviations: AI, atrichous isorhiza; AT, aboral tentacle; D, desmosome; MM, microbasic mastigophore; S, stenotele.

In contrast to the many studies using the adult polyps, only a few studies have been devoted to the larval stage. Instead of a planula larva, *Tubularia* hydroids produce the uniquely shaped actinula larva as the dispersive stage in their life cycle. Only preliminary studies have been carried out on the behavior and settlement process of the actinula larva (Pyefinch and Downing, 1949; Berrill, 1952; Hawes, 1958; Orlov and Marfenin, 1994), and a few ecological studies on larval recruitment have been performed (Lemire and Bourget, 1996; Nellis and Bourget, 1996; Walters and Wethey, 1996). Berrill (1952) and Hawes (1958) suggested that the temporary attachment of the actinula was achieved by nematocyst discharge from the aboral tentacle tips. However, the correlation between larval behavior, development, and nematocyte dynamics was not accurately described. They concluded that actinula development is direct (or nearly direct) development without any marked changes in larval behaviors, structures, and habits.

In this study, to understand actinular settlement, we extensively examined larval behavior, morphological transformation, and nematocyte dynamics in the actinula larvae of *Tubularia mesembryanthemum*. Along the Japanese coasts, dense colonies of this species can be observed throughout the year on artificial substrata used in aquaculture or in the cooling water systems of power plants. With the aid of microscopic and time-lapse video recording techniques, we observed that radical behavioral, morphological, and tentacular functional changes occurred during settlement of the actinula larvae. Nematocytes were important in larval attachment and metamorphosis, and their composition in the tentacles changed radically during this stage. These results contribute to an understanding of the development of actinula larvae and of nematocyte dynamics in the settlement of cnidarian larvae.

Materials and Methods

General observations on seasonal variation of colonies in the field

Colonies of *Tubularia mesembryanthemum* were collected from fishing nets, ropes, and floats near Nagai harbor in Sagami Bay (eastern Japan, 35°10′ N, 139°35′ E) and from near Sakurajima in Kagoshima Bay (southern Japan, 31°35′ N, 130°35′ E). At the former site, colonies of *Tubularia mesembryanthemum* were observed and surface water temperatures were measured once to four times a month between December 1993 and August 1996.

Colony maintenance and liberation of actinulae

Adult colonies were washed several times by shaking them in natural seawater immediately after collection, then transferred to our laboratory in cooled seawater (1 to 3 colonies per liter) in insulated containers. At the laboratory,

the colonies were repeatedly washed in filtered seawater (FSW), and predatory nudibranchs and the muddy tubes of amphipods were removed. The colonies were placed in 50- or 200-l tanks, either by hanging them with plastic-coated wires or by putting them in plastic baskets. Tanks were filled with coarsely filtered running seawater to which a water jet was applied from the side (water temperature, 16 ± 3 °C). About 10 female colonies were maintained with 1 or 2 males in a tank. Six times a week, the colonies were fed on newly hatched *Artemia* nauplii or on the nauplii reared on a diet of *Isochrysis galbana* (Haptophycea). Actinula larvae were obtained by placing female branches or polyps in small plastic baskets in 2- or 3-l beakers filled with fresh FSW (about 10 polyps/l)

Larvae were collected from the bottom of the beakers by callus pipettes, which have a large bore, and rinsed three times in fresh FSW. Larval age was defined as the period following liberation from the maternal gonophores. During microscopic observations of behavior, larvae were kept at a temperature of 19 ± 1 °C.

Observations on larval behavior and morphogenesis during settlement

After the actinula larvae were released from the maternal gonophores, their behavior and morphogenesis were observed on either clean or microbial-filmed glass petri dishes under a stereoscopic microscope. Microbial films were grown on deep (∅ 6 cm × h 6 cm) glass petri dishes by exposing the dishes to coarsely filtered running seawater for 1 day to 3 weeks. For larval settlement assays, 15 ± 3 larvae (< 8 h old) were placed in either clean or microbial-filmed glass petri dishes containing 40 ml of 0.22-μm-membrane-filtered seawater (0.22-μm FSW).

In addition, larvae were placed in a glass cell that was dipped in running seawater (0.5 ml/s) and the detailed process of morphogenesis was continuously recorded under an inverted microscope coupled to a time-lapse video disk recorder (Sony LVR-3000 A/N). Larval and post-larval dimensions were measured until stolon elongation occurred in all 30 individuals.

Scanning electron microscopy and histological observation

Larvae were sampled at three stages: at ages 2–4 h, at ages 24–28 h, and at settling. They were fixed in 4% neutralized formaldehyde in artificial seawater (ASW) and dehydrated in a graded series of ethanol. For scanning electron microscopy, the specimens were steeped three times in 100% *t*-butanol for 30 min, frozen at −20 °C overnight, freeze-dried in a Hitachi ES-2300 vacuum freeze dryer, coated with palladium-platinum in a Hitachi E-102 ion spatter-coater, and examined under a Hitachi S-2400 scanning electron microscope.

For histological observation under an optical microscope, the fixed and dehydrated specimens were embedded in Technobit 7100 resin (Heraeus Kulzer GmbH, Germany), sectioned at a thickness of 6 μm, and stained with hematoxylin-eosin.

Nematocyte composition and migration in the tentacles

The composition of nematocytes in the aboral tentacles or their rudiments was examined in star-shaped embryos, preactinulae before liberation, 2–4-h-old larvae, 24–28-h-old larvae, settling larvae, settled individuals, and juvenile polyps 2 days after settlement. After the gonotheca was cut open with a thin needle, the star-shaped embryos and preactinulae were picked up with the needle from the dissociated gonophores. Each specimen was placed on a glass slide to which a drop of approximately 200 mM Mg^{2+} ASW was added to prevent shrinkage of the aboral tentacles and discharge of the nematocysts. After 30 min, the specimen was covered with a cover slip. The number of nematocytes in the aboral tentacles (or the rudiments) of each of 10 specimens was counted under a Nomarski interference microscope.

To examine nematocyte migration, a 2–4-h-old larva was placed in a petri dish filled with FSW, and the basal protrusion and the aboral tentacle were held by gentle suction with micropipettes. Nematocyte migration through the aboral tentacle was recorded under an inverted microscope coupled with a time-lapse video disk recorder (Sony LVR-3000 A/N).

Statistical analysis

One-way analysis of variance (ANOVA) with Scheffé's test as a *post hoc* test was used to examine the significance of the changes in nematocyte composition and the effect of the microbial films on larval settlement. A significance level of $P < 0.05$ was used in all statistical analyses, which were performed using StatView version 5.0 (SAS Institute, Inc., USA).

Results

Seasonal variation of colonies in the field

Figure 1 shows the general succession of colonies of *Tubularia mesembryanthemum* on artificial substrata (vinylon nets, ropes, and plastic buoys) hanging from the set nets for fisheries near Nagai harbor during the period from December 1993 to August 1996. No colonies were seen inside the harbor, but colonies were often observed on the shadowed surfaces of fishing nets and ropes (around the depths of 3–10 m) and on the lower surfaces of buoys outside the harbor. They were also seen on the shell surface of the barnacles *Megabalanus rosa* and *M. volcano* and the mussel *Mytilus galloprovincialis* that settled on the artificial

substrata. Colonies of *T. mesembryanthemum* were found throughout the year; colony growth and degeneration was repeated in a cycle of 1 to 2 months, with new colonies appearing at new settlement sites. Large numbers of colonies were observed from November to July (surface water temperature, 12–26 °C), and fully matured colonies were dominant in a wide range of water temperature: February-March (12–14 °C), June-July (20–26 °C), and October-November (24–20 °C). Mature colonies reached maximum density in June and July (19–26 °C), covering almost all surfaces of the fishery's nets and ropes. These mature colonies degenerated rapidly, and colonies with only perisarcs were observed in August or September (surface water temperature, 26–30 °C).

Colony maintenance and actinula liberation

The colonies were basically dioecious, although bisexual polyps were occasionally observed; the form of the gonophores was very variable under laboratory culture conditions. Fertilized eggs developed to star-shaped embryos, preactinulae, and then to actinula larvae that were released from the maternal gonophore (Fig. 2A-D).

Maintenance and culture were dependent on the physiological state of the colonies collected; colonies without degeneration of the hydocaulus stayed mature for 1–2 months in running seawater and released actinula larvae repeatedly, every 1–2 weeks. The number of gonophores gradually decreased during the 2 months after collection.

A polyp released from 20 to 300 actinula larvae, depending on the degree of maturation. Fully matured female polyps released most of their larvae within the first 3 days from the beginning of release. Larvae released later were smaller and sometimes deformed. Larvae released early in the period were used for experiments, because some later-released larvae degenerated before stolon elongation.

Changes in larval behavior and morphology

Newly released actinulae had 4–12 (mainly 8 or 6) aboral tentacles and 4–7 oral tentacle rudiments (Fig. 2D). When larvae were placed in a 2-1 beaker filled with FSW so that their tentacles did not touch the surface of vessels, they showed a specific floating behavior with the oral pole turned downward and the tentacles held rigid and stretched backward (resembling the seed of a dandelion). However, as soon as a tentacle tip touched the substrata surface, the larvae moved some of their aboral tentacles up and some down (Fig. 2D). Larvae aged 2–8 h showed the following characteristics: aboral tentacle length, 600–1000 μm (mean $\pm$ SD, 792.7 $\pm$ 92.2); oral tentacle (rudiment) length, 13–53 μm (33.4 $\pm$ 10.6); body length, 233–367 μm (305.1 $\pm$ 33.2); body width, 200–310 μm (236.3 $\pm$ 29.3). About 12 h after liberation, the larvae began a repeated contraction and expansion of their bodies (Fig. 3A); the
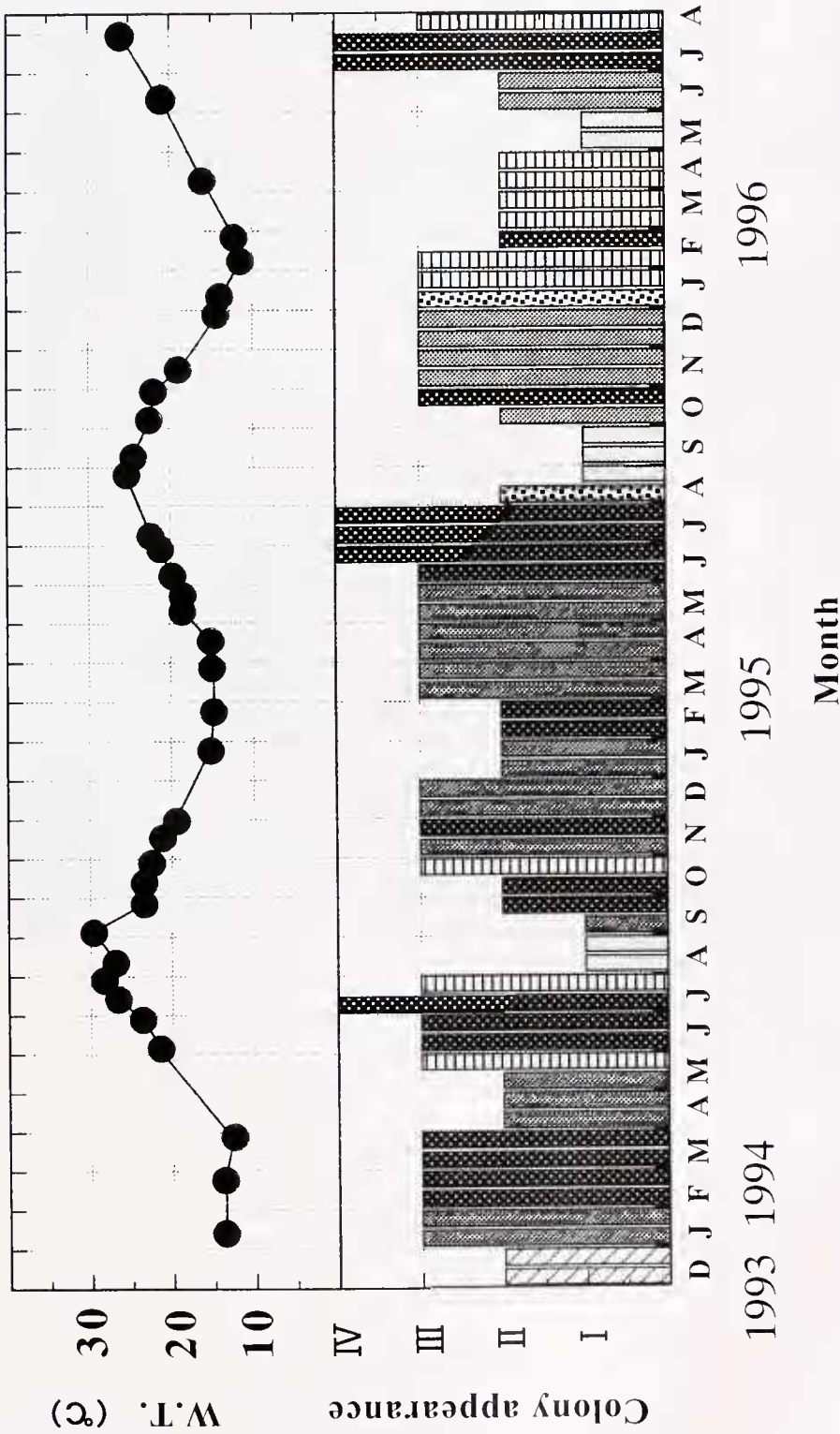
Figure 1. Seasonal variation of the colonies of *Tubularia mesembryanthemum* growing attached to the artificial substrata used in set nets for fisheries at Nagai harbor. from December 1993 to August 1996. Ranks of colony appearance shows the approximate number of observed colonies as I, <10; II, <50; III, ≥50; IV, colonies covered all surfaces (underwater) of most fisheries nets, ropes, and buoys.
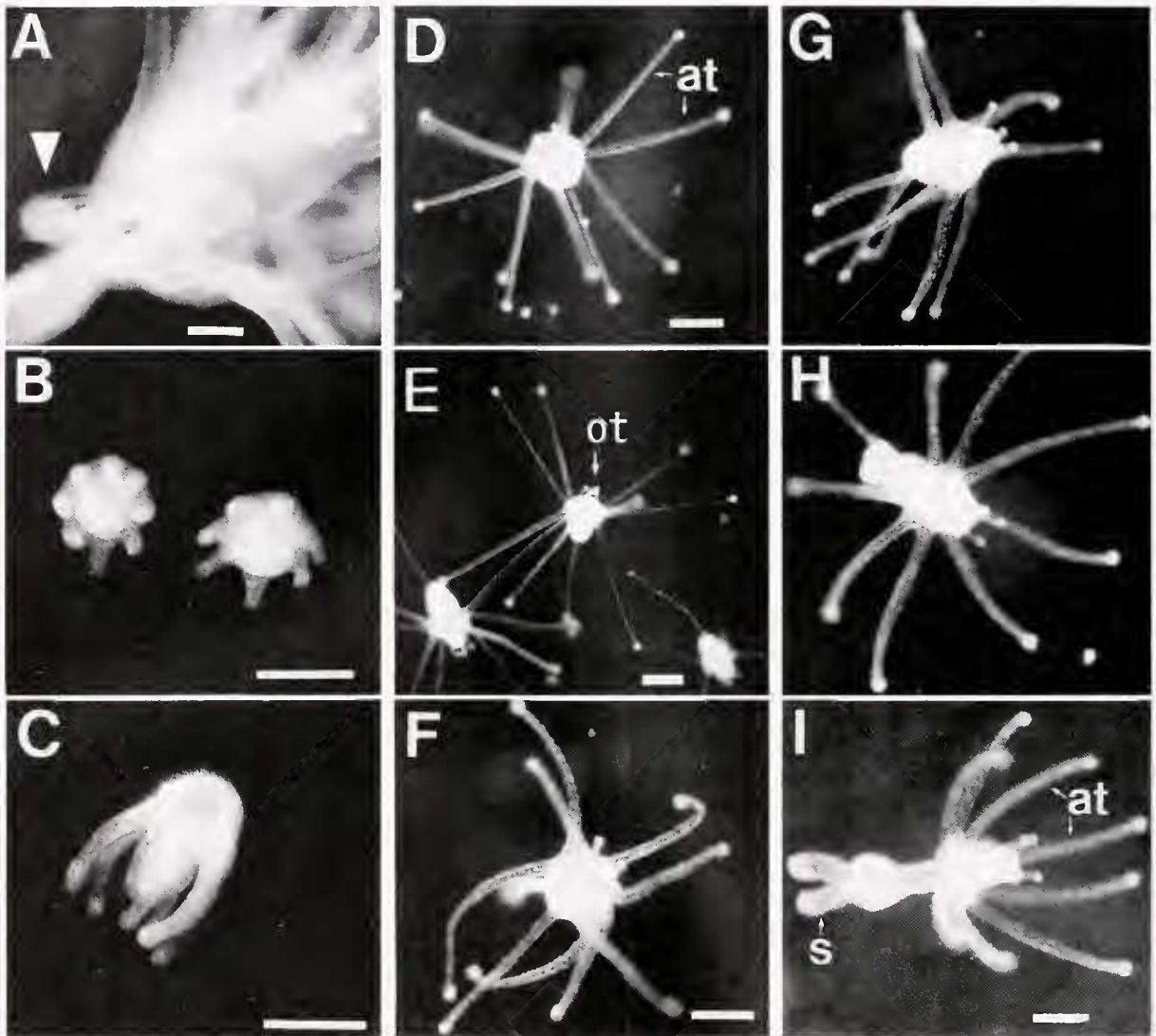
Figure 2. Development and behavior in an actinula larva of *Tubularia mesembryanthemum*. (A) Female polyp bearing actinulae (arrowhead); (B) star-shaped embryos; (C) preactinula (before liberation); (D) actinula larva newly released from maternal gonophore; (E) 1-day-old larva; (F and G) 1-day-old larva during settling behavior; (H) individual settled by the aboral pole (= basal protrusion); (I) stolon-elongated juvenile polyp 1 day after settlement. at = aboral tentacle(s), ot = oral tentacle(s), s = stolon. F–H are same magnification. Bars = 300 μm.

aboral tentacles, particularly the tips, became so sticky that they could not be pipetted away from the substrata, and they eventually formed a temporary attachment after coming in contact with a surface. Later the larvae often bent their tentacles onto the bottom surface and used them to move slowly on the bottom of the beakers.

One day after liberation, the aboral and oral tentacles elongated [aboral tentacles, 700–1050 μm (910.7 ± 102.4); oral tentacles, 53–120 μm (81.7 ± 16.9)], body length increased [280–500 μm (345 ± 52.1)], and the aboral pole (= basal protrusion which later became the location of permanent attachment) gradually began to extend downwards (Fig. 2E). Larvae then positioned themselves by

pressing the basal protrusion, composed of long columnar gland cells filled with secretory granules that stained strongly with eosin (Fig. 4A, B), onto the substrata. This action was followed by active sinuous movement and rubbing of the aboral tentacles (around the settlement site), accompanied by swaying of the body on the substrata surface, that was only observed during larval settlement (Fig. 2F, G; Fig. 3B).

Settlement was followed by release of the cement substance. Newly settled individuals temporarily raised their aboral tentacles upward, but if the vessel was shaken, they immediately moved some tentacles downward and touched the substrata surfaces. Then, the larvae irreversibly opened
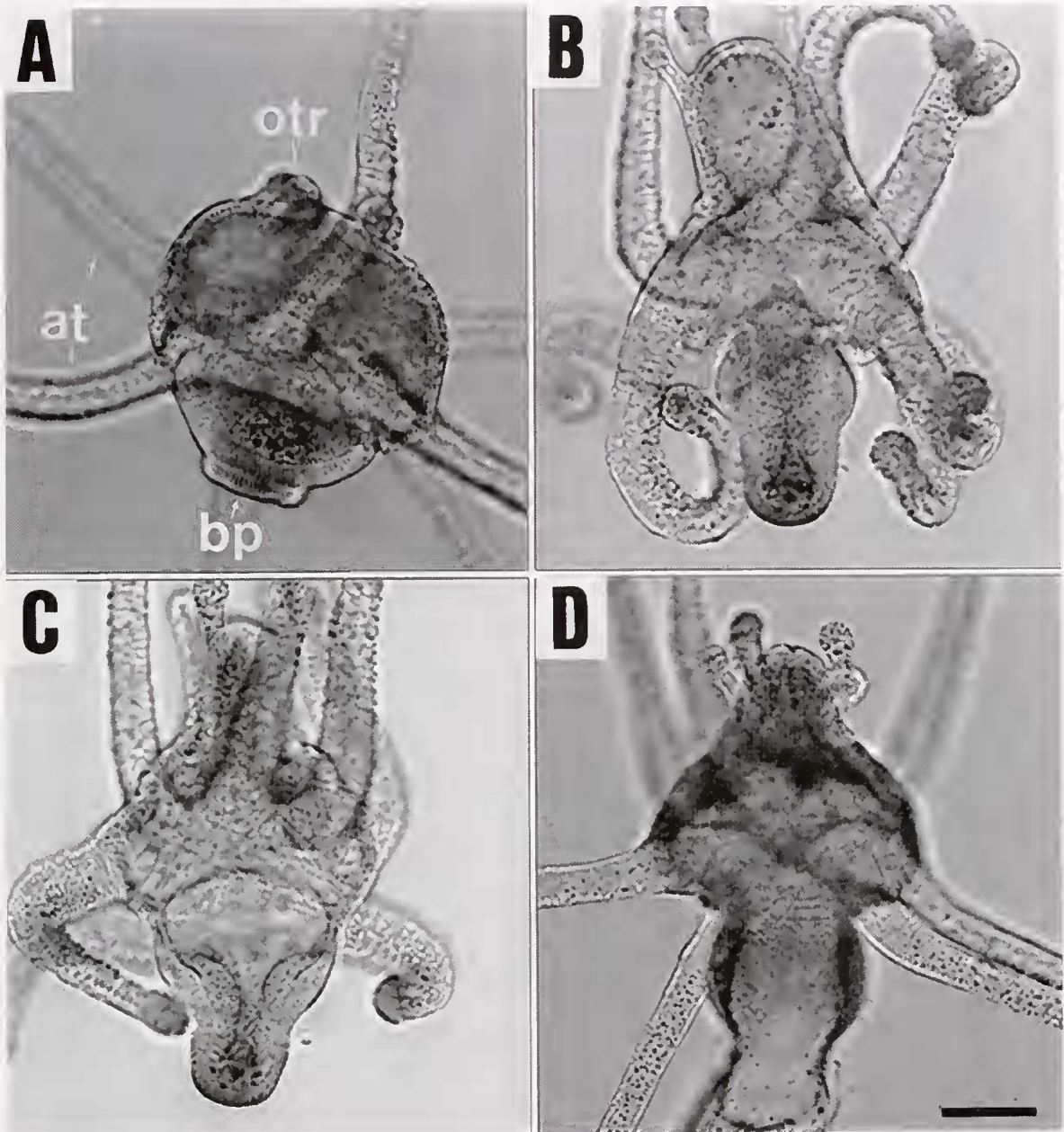
Figure 3. Morphological changes in the body of an actinula larva during settlement (time-lapse video images). (A) Larva undergoing repeated contraction and expansion of the body. (B) Larva beginning settling behavior with sinuous movement and rubbing of the tentacles to surface substrate; this phase was reversible to the temporary attachment phase. (C) Larva that has irreversibly opened the coelenteron in its aboral half to permanent settlement by the basal protrusion. (D) Stolon-elongated juvenile polyp. at = aboral tentacle(s), bp = basal protrusion, otr = oral tentacle rudiment(s). A–D are same magnification. Bar = 100 μm.

the coelenteron in the aboral half of the bodies and achieved permanent attachment at the basal protrusion (Fig. 3C). Subsequently, the stem and stolon differentiated and all tentacles extended upward (Fig. 2H, I; Fig. 3D). In these juvenile polyps, the cushion-like tissue disappeared into the endoderm of the aboral half, and many digestive gland cells developed in the endoderm of the oral half of the hydranth (Fig. 4C, D). The resulting structure contrasts with the well-developed cushion-like tissue in the endoderm of the aboral half of an actinula larva and the absence of digestive gland cells in the endoderm of the oral half of the larval body (Fig. 4A, B). Thus, morphological transformation to the juvenile polyps was completed.

When larvae (< 8 h old) were given clean glass surfaces
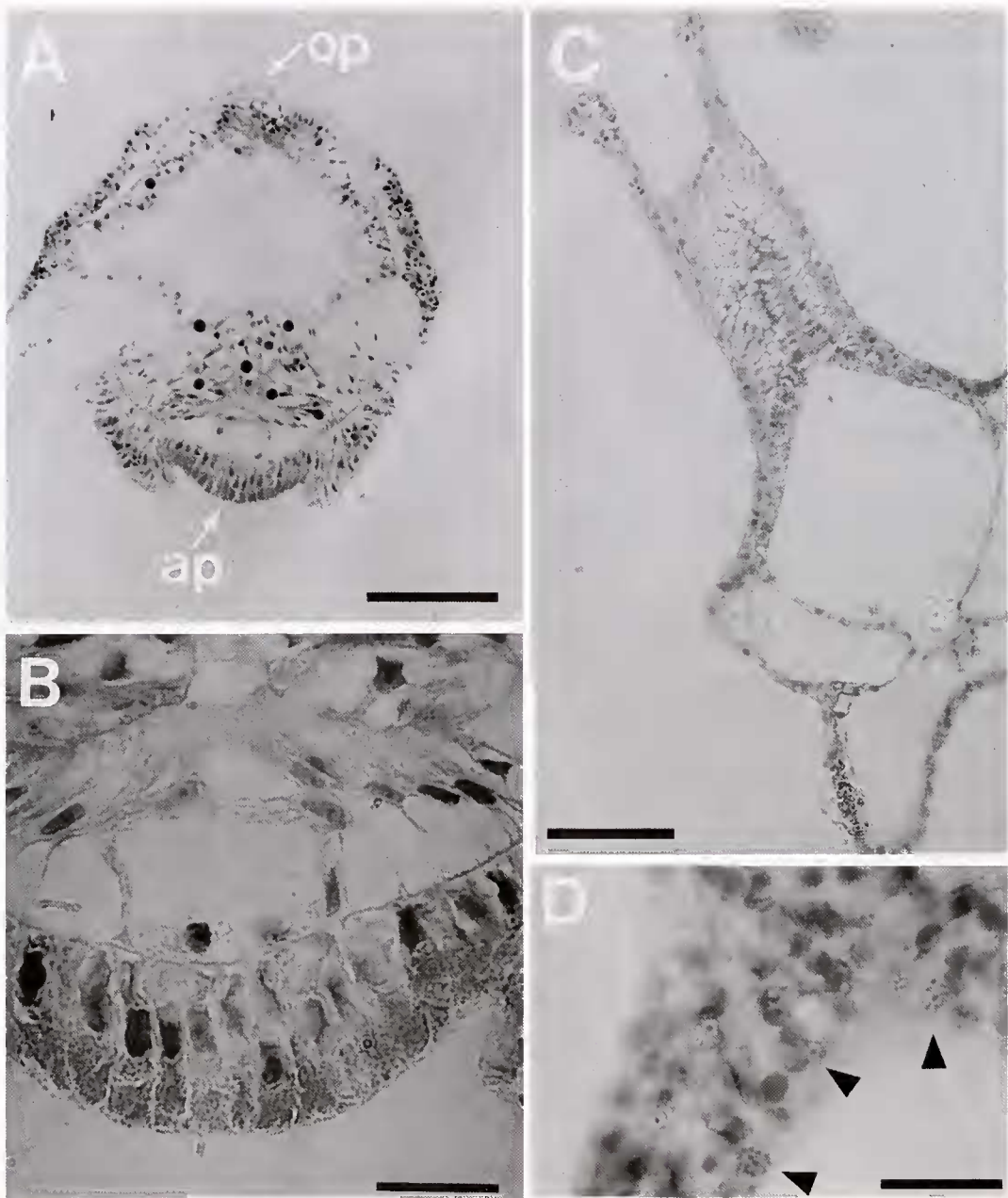
Figure 4. Longitudinal section of actinula larva (2–4 h old) and hydranth of juvenile polyp (1 day after settlement) stained with hematoxylin-eosin. (A and B) Actinula larva; (C and D) juvenile polyp. Long columnar gland cells filled with strongly eosinophilic granules were arranged in the ectoderm of the aboral pole (= basal protrusion) of the actinula larva, and the many digestive gland cells (arrowheads) were observed in the oral endoderm of the hydranth of the juvenile polyp. Bar = 100 μm (A, C) and 20 μm (B, D).

in still water (0.22-μm FSW), almost none of them initiated settling behavior within 16 h (about 24 h from the larval liberation); after 16 h, settled and stolon-elongated individuals represented only 1.5% ± 4.6% (mean ± standard deviation) of the total ($n = 26$). In most cases, settling behavior did not begin for 24 h, then the larvae usually started and stopped the behavior repeatedly and erratically; some larvae did not settle for more than 2 weeks.

In contrast, when they were exposed to surfaces that had acquired microbial films by being kept in running natural seawater for 2 to 3 weeks, < 8 h-old larvae began the settling behavior immediately after their tentacle tips touched the substrata. They then settled quickly, with stolon elongation; percent settled and stolon-elongated individuals after 16 h were 67.4% ± 25.8% ($n = 15$) when exposed to 2-week-old films, and 74.5% ± 15.8% ($n = 13$) in the case

of 3-week-old films. Thus, microbial films significantly ($P < 0.0001$) promoted actinular settlement. These larval responses did not occur without direct contact of the aboral tentacles to the microbial-filmed surface.

Three days after liberation, juvenile polyps showed the following characteristics: aboral tentacle length, 600–1300 μm (mean $\pm$ standard deviation $=$ 800.8 $\pm$ 187.2); oral tentacle length, 73–120 μm (89.0 $\pm$ 17.3); body length, 433–968 μm (660.9 $\pm$ 186.6); body width, 130–200 μm (170.9 $\pm$ 23.4).

### Discharge and printing of the tentacular nematocytes during larval settlement

Large numbers of *atrichous isorhizas* (AIs) were observed at the tips of the aboral tentacles in the pre-attachment stage (Fig. 5A, C). With time following larval liberation, the knobs of the aboral tentacle tips gradually became indistinct, and so sticky that repeated pipetting could not tear the tips from the substrata. These changes were accompanied by the transformation of the knob surfaces (the number of cnidocils decreased and the number of discharged tubes increased, Fig. 5B). Temporary attachment

was achieved by anchoring with the tubes of the discharged AIs to the substrata surface.

Observations with the inverted and scanning electron microscopes showed that the settling larvae discharged many AI nematocytes from the aboral tentacle tips in concert with their sinuous movement, active rubbing of the tentacles, and body-swaying on the glass surfaces during settlement. As a result, the discharged nematocytes were stuck around the settlement site (Figs. 6, 7). In contrast, temporary attachment was achieved by one or two tubes of the discharged AIs from a rigid tentacle; the sinuous tentacular behavior and the AI discharge and sticking (more than 50 from a tentacle) were peculiar to larval settlement (Figs. 6, 7) and thus were termed "nematocyte-printing behavior."

Dynamic changes in nematocyte composition during larval settlement

Figure 8 shows the changes in nematocyte composition of the aboral tentacles during larval development and behavior. No nematocytes were observed in the aboral tentacle rudiments of the star-shaped embryos. The preactinula had only small numbers of atrichous isorhizas (AIs) (20.5 $\pm$
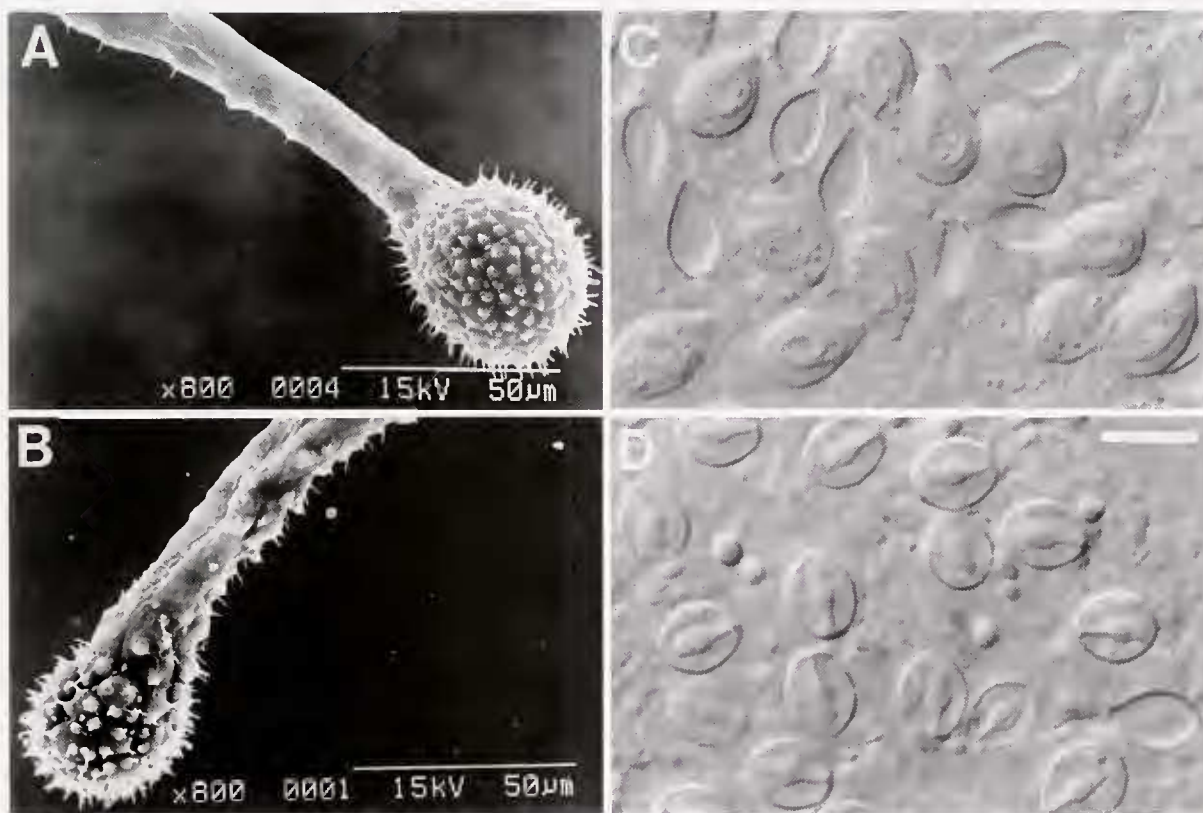
Figure 5. Scanning electron micrographs of aboral tentacle tips of actinula larvae, and Nomarski interference micrographs of nematocysts in the tentacle tip and body wall. (A) 2–4-h-old larva; (B) 1-day-old larva; (C) atrichous isorhiza nematocysts in the tentacle tip; (D) small stenotele nematocysts in the body wall (2–4-h-old larva). C and D are same magnification. Bar = 5 μm.
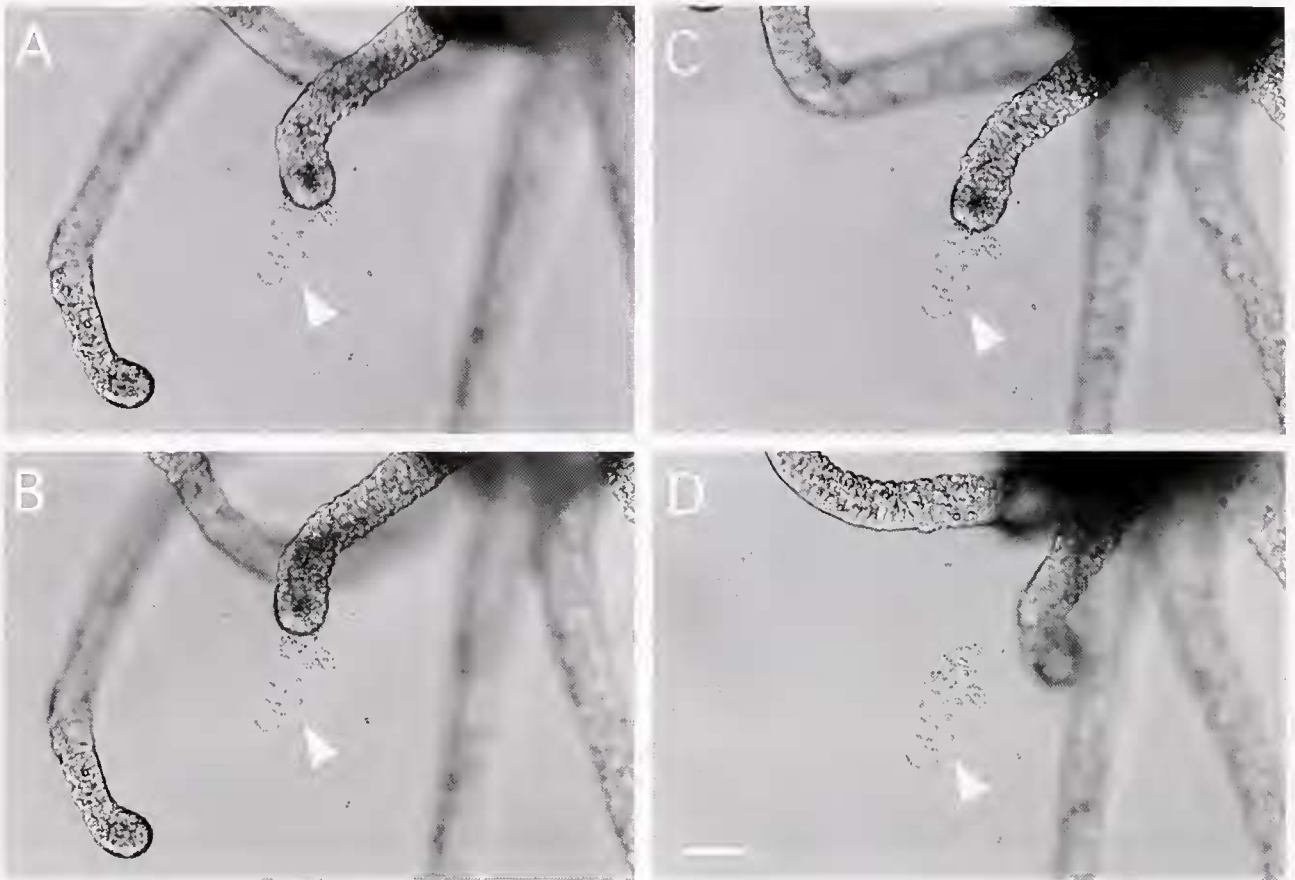
**Figure 6.**   Rubbing of the aboral tentacle tip with sinuous movement, followed by discharge and sticking of atrichous isorhiza nematocytes (arrowheads) on the glass surface during the settlement of an actinula larva. The elapsed time from A to D was about 5 min. A–D are same magnification. Bar = 50 μm.

1.9), which were not deployed in the tentacle tips. In the tentacles of 2–4-h-old larvae four types of nematocytes, namely atrichous isorhiza (AI), stenotele (S), desmoneme (D), and microbasic mastigophore (MM), were observed. About 170 AIs were deployed at a tentacle tip at this stage, while small numbers of S, D, and MM were observed along an aboral tentacle (AI, 168.6 ± 41.3; S, 34.5 ± 13.0; D, 51.8 ± 23.9; MM, 11.8 ± 6.5; the total, 266.7 ± 64.2). In contrast, no AIs but numerous small Ss were present in the body walls of these larvae (Fig. 5). In 24–28-h-old larvae, the number of AIs in the tentacles gradually decreased, while other types of nematocytes (S, D, and MM) increased in number (AI, 82.9 ± 26.3; S, 101.3 ± 20.8; D, 95.3 ± 18.8; MM, 22.3 ± 8.5; the total, 301.8 ± 45.9). During pre-settlement and post-settlement stages, S and D nematocytes actively migrated in the ectoderm from the body wall to the tentacle tips at an speed of 6.0–15.4 μm/min (9.1 ± 5.4) (Fig. 9), which led to dynamic change in the tentacular nematocyte composition. Newly settled individuals that did not develop to hydrocaulus and hydrorhiza elongation retained small numbers of AIs in the aboral tentacle tips (AI, 16.1 ± 13.1; S, 199.3 ± 60.9; D, 168.6 ± 54.7; MM,

35.9 ± 16.5; the total, 419.8 ± 71.8). In polyps of 2 days after settlement, in which all aboral tentacles extended upward completely and the hydrocaulus was so long that the aboral tentacles could not touch the substrata, AI nematocytes disappeared and the aboral tentacles contained many nematocytes of three types (S, D, MM) (AI, 1.6 ± 3.3; S, 269.2 ± 53.8; D, 212.6 ± 42; MM, 32.3 ± 7.8; the total, 515.6 ± 81.8).

From 2–4-h-old larvae to juvenile polyps, the number of AIs decreased significantly (Table 1); however, the number of AIs in an aboral tentacle of the newly settled individuals varied very much (from 0 to 40). The increase in the number of Ss, Ds, and MMs was statistically significant (Table 1). The aboral tentacles of adult polyps had many holotrichous isorhiza-like nematocytes with S, D, and MM nematocytes.

When larvae were placed in clean glass petri dishes at a low density (1 ind./40 ml 0.22-μm-FSW), some actinula larvae remained floating or repeated temporary attachment for more than 2 weeks. These larvae retained many AIs in the aboral tentacle tips. When the aboral tentacle tips of the newly released actinula larvae were cut off, the tips were regenerated and AIs were deployed within 2 to 3 days; this
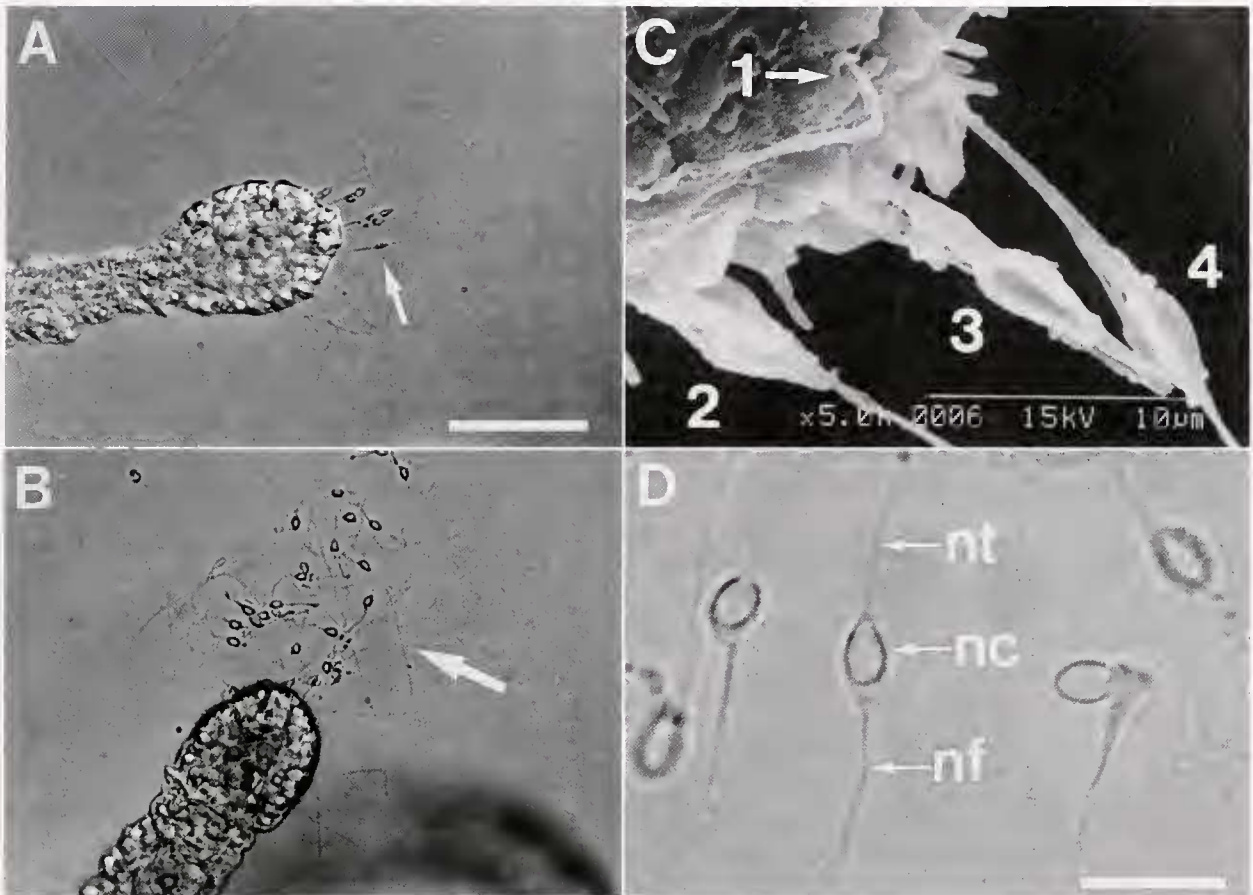
Figure 7. Atrichous isorhiza (At) nematocytes discharged from the aboral tentacle tips during nematocyte-printing behavior. (A and B) Nomarski micrographs: many Ats (arrows) were discharged from the tentacle tip, resulting in "printing" around the settlement site. (C) Scanning electron micrograph: different phases from At nematocyst discharge (1) to nematocyte exclusion (4) from the epithelial tissues of the tentacle tip (at about same phase as in A). (D) At nematocytes printed on glass surface. nc = nematocyst capsule, nf = nematocyte filament, nt = nematocyst tubule. A and B are same magnification. Bar = 50 μm (A, B) and 10 μm (D).

was followed by temporary attachment and larval settlement. In contrast, when the tentacle tips of the juvenile polyps were cut off, regeneration and deployment of Ss occurred in the aboral tentacles.

Discussion

Seasonal variation of colonies and liberation of actinulae

Field observations showed that both asexual and sexual reproduction of *Tubularia mesembryanthemum* continue in a wide range of water temperatures (surface water temperatures ranged from 12 to 26 °C) from October to July in the vicinity of Nagai coast. Supplies of larvae seemed to be most abundant in the range from 18 to 22 °C. Mature colonies were most dense from June to July, and appeared to degenerate within 1 or 2 months on certain substrata. Colonies appeared from late September to October, possibly regenerating from the once-degenerated stolons. Frequent

appearance of colonies on the shadowed surfaces of artificial substrata outside the harbor suggests the importance of certain kinds of microbial films and of water currents in actinular settlement followed by colony growth. Defining the reproductive cycle of *T. mesembryanthemum*, which is beyond the scope of this paper, would require tracking the progression of individual colonies, as Hughes (1983) did for *T. indivisa*.

### Changes in larval behavior and morphology during settlement

Several researchers preliminarily reported on the settlement behavior of actinula larvae of *Tubularia* spp. (Pyefinch and Downing, 1949; Berrill, 1952; Hawes, 1958; Lemire and Bourget, 1996), concluding that, because of their direct development, these larvae should be considered merely hydranths with rudimentary stalks, or only juvenile
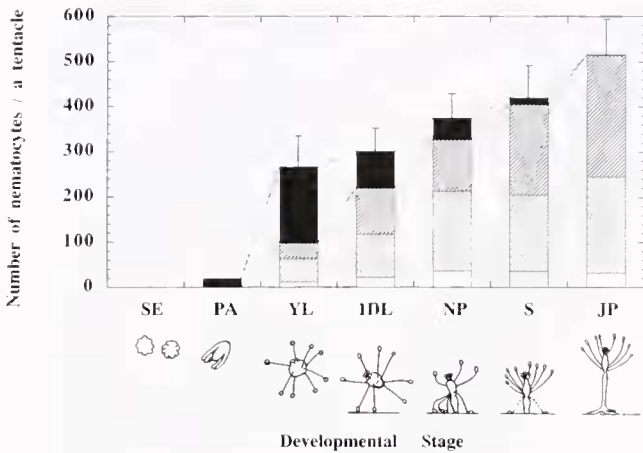
Figure 8. Changes in nematocyte composition of the aboral tentacles during larval development: ■ atrichous isorhizas, ▨ stenoteles, □ desmonemes, □ microbasic mastigophores. SE, star-shaped embryos; PA, preactinulae; YL, young (2–4-h-old) larvae; 1DL, 1-day (24–28 h)-old larvae; NP, nematocyte-printing larvae; S, newly settled individuals; JP, juvenile polyps 2 days after settlement. Vertical bars = standard deviation of total numbers of nematocysts in a tentacle.

polyps. In contrast, our observations revealed that the liberated actinula larvae underwent marked behavioral changes that could be divided into the following phases: floating (or sinking), temporary attachment, nematocyte-printing, and settlement followed by stolon elongation. In parallel with these behavioral changes, we observed functional changes in the aboral tentacles (from floating or temporary attachment to feeding or defense) and physiological changes in the body (disappearance of the cushion-like tissues of the aboral endoderm and development of digestive gland cells of the oral endoderm). In addition to these settlement processes, larvae whose settlement was delayed retained many atrichous isorhizas (AIs), and larvae whose tentacle tips had been cut off showed no settling behavior until the tips and the AI nematocytes regenerated. These results show that the settlement of actinulae occurs through coordination of the aboral tentacles and the basal protrusion, and that nematocyte-printing behavior is both peculiar to and indispensable for settlement and morphogenesis of the actinula larvae of *T. mesembryanthemum*. The settlement process of the actinula larvae thus proved to involve radical
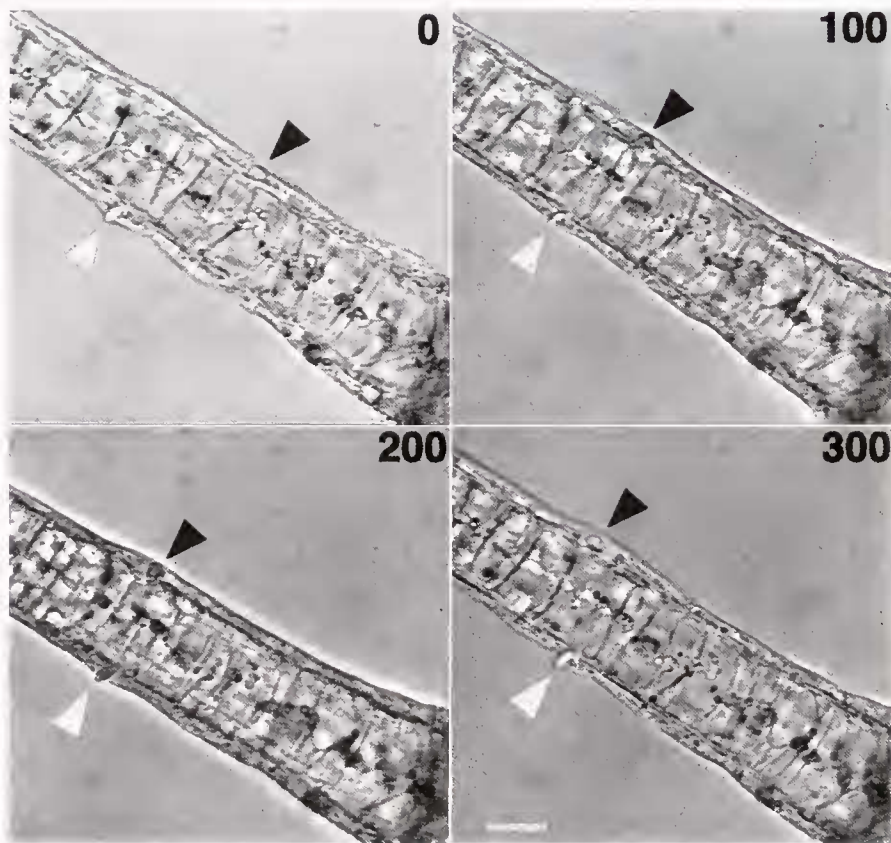
Figure 9. Migrating nematocyte along the epithelium of the aboral tentacle from base to tip of the tentacle before larval settlement. The nematocyte migrated at a speed of 6–15.4 μm/min (average speed of approximately 9 μm/min) toward the tentacle tip. Black arrowhead indicates the migrating nematocyte (probably stenotele), and white arrowhead indicates the deployed nematocyte (probably microbasic mastigophore). Numbers at upper right of each image show the elapsed time (seconds). Every image is the same magnification. Bar = 20 μm.

Table 1

Results of ANOVA (Scheffé's test, significance level $P < 0.05$*) to access the change in nematocyte composition of aboral tentacles from 2–4-h-old larvae to juvenile polyps*

Stages	Type of nematocyte				
	Atrichous isorhizas	Stenoteles	Desmonemes	Microbasic mastigophores	Total number of nematocysts
2–4h-old larvae, 1-day-old larvae	<0.0001*	0.0048*	0.0891	0.2101	0.7726
2–4h-old larvae, Nematocyst-printing	<0.0001*	0.0015*	<0.0001*	<0.0001*	0.0092*
2–4h-old larvae, Just-settled	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*
2–4h-old larvae, Juvenile polyps	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*
1-day-old larvae, Nematocyst-printing	0.0939	0.9855	0.0016*	0.1380	0.2792
1-day-old larvae, Just-settled	<0.0001*	<0.0001*	0.0003*	0.0498*	0.0012*
1-day-old larvae, Juvenile polyps	<0.0001*	<0.0001*	<0.0001*	0.2927	<0.0001*
Nematocyst-printing, Just-settled	0.0548	0.0004*	0.9933	0.9999	0.6469
Nematocyst-printing, Juvenile polyps	0.0019*	<0.0001*	0.3347	0.9479	0.0005*
Just-settled, Juvenile polyps	0.5643	0.0003*	0.0289*	0.9219	0.0029*

Numbers show P values. Asterisks (*) indicate significant differences.

morphogenetic reorganization. This complex series of behavioral and morphological changes during actinular settlement has not been reported previously.

Furthermore, the coordination of nematocyst discharge from the tentacle tips and cement secretion from the basal protrusion suggests that a physiologically complex set of internal signals controls actinular attachment and morphogenesis.

Nematocyte-printing behavior as active metamorphic event

The role of nematocytes in the temporary attachment of cnidarian larvae has been partially described by others (Hawes, 1958; Teitelbaum, 1966; Donaldson, 1974; Mariscal, 1974; Chia and Crawford, 1977; Chia and Bickell, 1978; Namikawa *et al.*, 1993); however, the correlation between larval settlement, morphogenesis, and nematocyte dynamics has not been accurately defined.

We found that actinula larvae attached temporarily to the substrata by AI discharge, and that the function of the larval tentacles changed irreversibly—from temporary attachment to feeding (and defense)—after larval settlement. Composition of the nematocytes in larval tentacles changed dramatically—from AI dominance to S/D dominance—after nematocyte-printing behavior and in parallel with the functional changes in the larval tentacles. AIs were used for temporary attachment of the actinula larvae, whereas Ss, Ds, and MMs were apparently used by the polyps for feeding or defense, as has been suggested for other species (Ewer, 1947; Mariscal, 1974; Chia and Bickell, 1978; Purcell and Mills, 1988; Tardent, 1995; Östman *et al.*, 1995). Moreover, actinula larvae whose tentacle tips had been cut off regenerated the AIs and then exhibited nematocyte-printing behavior followed by larval settlement.

Thus, AI nematocyte-printing behavior may be recognized as metamorphic behavior responsible for irreversible changes in aboral tentacle function from temporary attachment to feeding and defense. Whereas Berrill (1952) and Hawes (1958) concluded that actinulae of *Tubularia* are merely juvenile polyps and not really "true" larvae, the present study clearly shows that the actinula larvae of *T. mesembryanthemum* are not merely juvenile polyps but "true" larvae specialized for dispersal and attachment as part of the process of indirect development.

In addition, we demonstrated that actinula settlement was promoted by direct contact with microbial films formed on the substrata surface, and that settlement-delayed larvae retained many AIs in their aboral tentacles. These results suggest that actinular settlement is determined by close correlation between larval aging and nematocyte migration (as endogenous environment) and substrata surface environment (as exogenous environment). Further investigation is required to clarify the mechanisms of actinular settlement, though our previous biophysical studies revealed the importance of external and internal Ca^{2+} in larval settlement (Yamashita *et al.*, 1996, 1997; Kawaii *et al.*, 1997, 1999).

This report, which reveals that nematocyte-printing behavior is an active metamorphic event and that nematocyte dynamics is synchronously involved in the attachment and morphogenesis of actinula larvae, provides new insights into the attachment and morphogenesis of cnidarian larvae.

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